FINAL SCRIPT: APPROVED FOR FILMING

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Title: Plant Sample Preparation for Nucleoside/Nucleotide Content Measurement with An HPLC-MS/MS

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interview Statements are read by JoVE's voiceover talent.

4. **Filming location:** Will the filming need to take place in multiple locations? **Yes**

   If **Yes**, how far apart are the locations? **500 meters**

Current Protocol Length

Number of Steps: 11
Number of Shots: 25

Introduction

Note: VO talent please record all the interview statement

1. **Introductory Interview Statements**

1.1. Nucleosides and nucleotides are central metabolic components in all living organisms. The metabolism of them is required for cell development. This approach provides a powerful tool for these metabolites' quantification.

1.1.1. LAB MEDIA: Figure 1 A.

1.2. This method allows rapid and accurate quantification of nucleosides and nucleotides in plants. Complete sample pre-treatments and LC-MS-MS analyses take about 2 days for a set of 10 to 20 samples. This method can be applied to all plants and even other organisms.

1.2.1. *2.3.1, 2.3.2.*

Introduction of Demonstrator on Camera

1.3. Demonstrating the procedure will be Changhua Zhu, an associate professor from the Chen laboratory.

1.3.1. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Protocol

2. Nucleosides/Nucleotides extraction

2.1. To grow the plants, ensure that *Arabidopsis* seeds are sterilized in 70% ethanol for 10 minutes and sowed on agar plates with one-half-strength Murashige and Skoog nutrients **[1]**.

2.1.1. Talent placing sterilized seeds on a plate.

2.2. Incubate the plates in the dark at 4 degrees Celsius for 48 hours **[1]**, then transfer them into a controlled growth chamber under 16 hours of light at 22 degrees Celsius and 8 hours of dark at 20 degrees Celsius **[2]**.

2.2.1. Talent putting the plates to incubate in the dark.

2.2.2. Talent putting the plates in the growth chamber.

2.3. Harvest 100 milligrams of 2-week seedlings **[1]** and freeze them in liquid nitrogen for metabolite extraction **[2]**. Grind 100 milligrams of frozen plant tissues with 7 to 8 steel beads in a pre-cooled mixer mill for 5 minutes at a frequency of 60 Hertz **[3]**. *Videographer: This step is important!*

2.3.1. Talent harvesting the seedlings.

2.3.2. Talent freezing the seedlings in liquid nitrogen.

2.3.3. Talent grinding the plant tissue.

2.4. Prepare the extraction solution, which contains methanol, acetonitrile, and water at a ratio of 2 to 2 to 1 **[1]**. Resuspend the homogenized materials with 1 milliliter of the extraction solution **[2]**. *Videographer: This step is important!*

2.4.1. Talent preparing the extraction solution.

2.4.2. Talent resuspending the plant tissue.

2.5. Centrifuge the resulting solution at 12,000 x *g* for 15 minutes at 4 degrees Celsius **[1]**, then transfer 0.5 milliliters of the suspension to a new 1.5-milliliter tube **[2]** and freeze it in liquid nitrogen **[3]**. Evaporate the frozen sample in a freeze dryer **[4]** and resuspend it in 0.1 milliliter of 5% acetonitrile and 95% water **[5]**. *Videographer: This step is difficult and important!*

2.5.1. Talent putting the solution in the centrifuge.

2.5.2. Talent transferring the supernatant to a tube.

2.5.3. Talent freezing the sample tube.

2.5.4. Talent evaporating the sample in a freeze dryer.

2.5.5. Talent resuspending the sample.

2.6. Centrifuge the resulting solution at 40,000 x *g* for 10 minutes at 4 degrees Celsius **[1]**. Load the supernatant into a vial for LC-MS/MS measurement **[2]**.

2.6.1. Talent putting the sample in the centrifuge and closing the lid.

2.6.2. Talent transferring the supernatant into a LC-MS/MS vial.

3. LC-MS/MS measurement

3.1. Prepare a 10 millimolar ammonium acetate buffer by dissolving 1.1 grams of ammonium acetate in 2 liters of double deionized water **[1-TXT]**. Adjust the pH to 9.5 with 10% ammonium and acetate acid **[2]**. *Videographer: This step is important!*

3.1.1. Talent preparing ammonium acetate buffer. **TEXT: Mobile phase A**

3.1.2. Talent adjusting the pH.

3.2. Prepare 2 liters of ultrapure 100% methanol for nucleosides measurement **[1-TXT]**. Then, prepare 2 liters of ultrapure 100% acetonitrile for nucleotides measurement **[2-TXT]**.

3.2.1. Talent preparing the methanol. **TEXT: Mobile phase B1**

3.2.2. Talent preparing the acetonitrile. **TEXT: Mobile phase B2**

3.3. Inject 0.02 milliliters of the pre-treated metabolite extraction of each previously prepared sample into a HPLC system with binary pumps coupled with a triple quadrupole mass spectrometer **[1]**.

3.3.1. Talent injecting an extraction into the HPLC system.

3.4. To generate standard calibration curves, pool 6 sample extractions together and vortex the mixture **[1]**. Then, aliquot it to six extractions again to obtain each background **[2]**. *Videographer: This step is difficult and important!*

3.4.1. Talent pooling extractions together.

3.4.2. Talent aliquoting the extractions.

3.5. Add six different concentrations of each standard to these six extractions, respectively **[1]**, and inject them one by one into the HPLC system **[2]**. Record the peak areas of each standard at different concentrations via the mass transitions **[3]**.

3.5.1. Talent adding a standard to an extraction.

3.5.2. Talent injecting the extraction into the HPLC system.

3.5.3. Talent recording the peaks.

Results

4. Results: Identification of N^1-methyladenosine by mass transition

4.1. This protocol was used to identify and quantify N^1-methyladenosine *(pronounce 'N one methyl-adenosine')*, a known modified nucleoside, in 2-week-old *Arabidopsis* wild type seedlings **[1]**.

4.1.1. LAB MEDIA: Figure 2.

4.2. The mass spectrometry profile indicated that the product ions generated from the N^1-methyladenosine standard are at 150 and 133 MZ-ratios **[1]**, and the same profile was observed in the Columbia zero extraction **[2]**.

4.2.1. LAB MEDIA: Figure 2. *Video Editor: Emphasize A.*

4.2.2. LAB MEDIA: Figure 2. *Video Editor: Emphasize B.*

4.3. Due to high abundance of the product ion of 150 MZ, the mass transition of 282.1 to 150 was selected for the N^1-methyladenosine identification **[1]**. The retention time of the target peak was 7.05 minutes **[2]**, same as the retention time of N^1-methyladenosine standard **[3]**.

4.3.1. LAB MEDIA: Figure 3.

4.3.2. LAB MEDIA: Figure 3. *Video Editor: Emphasize B.*

4.3.3. LAB MEDIA: Figure 3. *Video Editor: Emphasize A.*

4.4. A concentration series of N^1-methyladenosine standards was added into six sample extractions **[1]**. The standard samples were injected into the LC-MS/MS and the increased peak areas of N^1-methyladenosine were plotted against the nominal concentrations of the standards **[2]**.

4.4.1. LAB MEDIA: Figure 4 A.

4.4.2. LAB MEDIA: Figure 4 B.

Conclusion

Note: VO talent please record all the interview statement

5. Conclusion Interview Statements

5.1. The six equal background extractions are important for making an accurate and repeatable calibration curve for the quantification.

5.1.1. *3.4.1, 3.4.2.*

5.2. Our method can be applied to explore metabolic pathways in plants. By comparing the metabolite profiles between wild type and loss-of-function mutants, the metabolic flux could be drawn.

5.2.1. *3.5.3.*